Research Article

A Monoclonal Antibody that Binds Anionic Phospholipids on Tumor Blood Vessels Enhances the Antitumor Effect of Docetaxel on Human Breast Tumors in Mice

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Abstract

Anionic phospholipids, principally phosphatidylserine, become exposed on the external surface of viable vascular endothelial cells in tumors, providing an excellent marker for tumor vascular targeting. We recently raised an IgG monoclonal antibody, 3G4, which binds to anionic phospholipids in a β2-glycoprotein I–dependent manner. It inhibited tumor growth in a variety of rodent tumor models by stimulating antibody-dependent cellular cytotoxicity toward tumor vessels. In the present study, we tested the hypothesis that docetaxel, which is known to have antivascular effects on tumors, might induce exposure of anionic phospholipids on tumor vasculature and, thus, enhance the antitumor activity of 3G4. Treatment of human umbilical vascular endothelial cells with subtoxic concentrations of docetaxel (20 pmol/L) *in vitro* caused anionic phospholipids to be externalized without inducing apoptosis. Docetaxel treatment of mice increased the percentage of tumor vessels that expose anionic phospholipids from 35% to 60%. No induction of phosphatidylserine was observed on vessels in normal tissues even after systemic treatment with docetaxel. Treatment of mice bearing orthotopic MDA-MB-435 human breast tumors with 3G4 plus docetaxel inhibited tumor growth by 93%. Treatment of mice bearing disseminated MDA-MB-435 tumors with 3G4 plus docetaxel reduced the average number of tumor colonies in the lungs by 93% and half the animals did not develop tumors. In both tumor models, the antitumor effect of the combination was statistically superior ($P < 0.01$) to that of docetaxel or 3G4 alone. Combination therapy reduced the tumor vessel density and plasma volume in tumors to a greater extent than did the individual drugs. The combination therapy was no more toxic to the mice than was docetaxel alone. These results indicate that, as an adjuvant therapy, 3G4 could enhance the therapeutic efficacy of docetaxel in breast cancer patients. (Cancer Res 2005; 65(10): 4408-16)

Introduction

Anionic phospholipids are largely absent from the surface of resting mammalian cells, including the vascular endothelium. Phosphatidylserine, which is the most abundant anionic phospholipid of the plasma membrane, is tightly segregated to the internal surface of the plasma membrane under normal conditions in most cell types (1, 2). Exposure of phosphatidylserine on the external surface of cells occurs during apoptosis and necrosis, and also on viable cells during cell activation (3) and transformation (4).

Recently, we used specific antibodies to show that anionic phospholipids become exposed on vascular endothelial cells in tumors but not in normal tissues (5–7). The vascular endothelial cells in tumors that expose anionic phospholipids are mc viable. They are morphologically intact, lack markers of apopt and line vessels that are functional at transporting blood solutes. Stress conditions, such as hypoxia, acidity, throm inflammatory cytokines, and reactive oxygen species in the tu microenvironment, may be responsible for externalization anionic phospholipids on the vascular endothelium in tumors The externalized anionic phospholipids are among the n specific markers of tumor vasculature yet identified. They present on tumor endothelial cells in various solid tum including metastatic tumors and drug-resistant tumors, and on the luminal side of tumor endothelium, which is fr accessible for binding by targeted drugs (6, 7). They undetectable on vessels in normal tissues, including the ovary and kidney, where angiogenesis or permeability markers are up-regulated (8). Annexin V, which has been used successfully for imaging anionic phospholipids in thrombi *in vivo* (9), also stained tumor vascular endothelium in a manner that was indistinguishable from the antiphosphatidylserine antibodies.

We recently raised an IgG3 monoclonal antibody, 3G4, that binds anionic phospholipids in a β2-glycoprotein I–dependent manner. After injection into tumor-bearing mice, 3G4 localizes specifically to complexes of anionic phospholipids and β_2-glycoprotein I on tumor vessels and mediates the binding of host effector cells to the tumor vascular endothelium. This is followed by damage to tumor vessels, a reduction in tumor vascularity, tumor necrosis, and retardation of tumor growth in multiple models (10). Antibody-dependent cellular cytotoxicity and antibody-dependent macrophage phagocytosis are implicated in the damaging effect of 3G4 on tumor vessels. 3G4, as a single agent, suppressed tumor growth by 60% to 90% in mice bearing orthotopic human breast tumors, Hodgkin's tumors, or a syngeneic mouse fibrosarcoma (10). Toxicity to the mice was not observed.

In the present study, we sought to determine whether combining 3G4 with docetaxel might enhance the antitumor activity of both drugs. There were several reasons to believe this would be the case. First, fewer than half (10-40%) of endothelial cells in solid tumors in mice have exposed anionic phospholipids. Docetaxel, which directly induces externalization of anionic phospholipids on several cell types (11, 12), might increase their exposure on tumor vessels and amplify the target for 3G4. Second, docetaxel might indirectly induce exposure of anionic phospholipids on tumor vasculature

Note: P.E. Thorpe and X. Huang are consultants to and have equity interest in Peregrine Pharmaceuticals, Inc.

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Exhibit J

by destroying tumor cells, stimulating lymphocyte infiltration, and causing local generation of reactive oxygen species. Third, the tumor cell killing patterns of 3G4 and docetaxel are complementary. 3G4, like other vascular targeting agents (VTA), is most effective against cells in the tumor core, which are oxygen-starved, nonproliferating, and resistant to chemotherapy, whereas docetaxel is most effective against the oxygenated, dividing tumor cells in the outer rim of the tumor (13, 14). Finally, the toxicity profile of VTAs is distinct from that of most other anticancer agents; thus, combinations of VTAs with conventional chemotherapy drugs are less likely to be more toxic than either agent alone. Antivascular agents combined with conventional chemotherapeutic drugs have shown additive or even synergistic antitumor activity in numerous studies in mice (15–18).

Docetaxel is a microtubule-binding drug of the taxane family with a wide spectrum of antitumor activity (19). It is one of the most effective drugs available for the treatment of breast, ovarian, and prostate cancers (20–23). It is becoming clear that docetaxel has multiple cellular targets, including apoptotic, angiogenic, and gene expression processes (24). Promising clinical results have been obtained using docetaxel in combination with other naked monoclonal antibodies, including herceptin (25), cetuximab (26), and bevacizumab (27).

We show here that 3G4 has enhanced antitumor activity when given with docetaxel to treat established (orthotopic) and disseminated human breast tumors in mice. The improvement derives, at least in part, from amplification by docetaxel of the phospholipid target for 3G4 on tumor vessels. The combination was no more toxic than was docetaxel alone. The 3G4/docetaxel combination merits further scrutiny as a potential treatment for human cancer.

Materials and Methods

Reagents. 3G4, a mouse IgG3 monoclonal antibody directed against anionic phospholipids, was raised in this laboratory and characterized as previously described (10). 3G4 binds to phosphatidylserine, phosphatidic acid, phosphatidylinositol, and phosphatidylglycerol but not to phosphatidylethanolamine, phosphatidylcholine, or sphingomyelin. Binding of 3G4 to anionic phospholipids is dependent on β_2-glycoprotein I. Antibodies for immunohistochemistry were purchased from commercial sources. Rat anti-mouse CD31 was from BD PharMingen (San Diego, CA). Peroxidase-conjugated goat anti-rat IgG (H + L) and Texas red–conjugated goat anti-rat IgG were from Jackson ImmunoResearch Lab (West Grove, PA). A hybridoma secreting monoclonal mouse IgG3, κ antibody against a *Babesia bovis* antigen, was obtained from the American Type Culture Collection (Rockville, MD). This monoclonal antibody is referred to herein as BBG3. BBG3 was used as a negative control for 3G4.

Detection of anionic phospholipids on human umbilical vascular endothelial cell and MDA-MB-435 cells *in vitro*. To induce exposure of anionic phospholipids, cells were grown on eight-well chamber slides to ~70% confluence. The cells were then treated with vehicle or docetaxel (20 pmol/L) in the presence of 3G4 or control antibody BBG3 (10 μg/mL) for 24 hours. Exposure of anionic phospholipids was then determined by live cell immunofluorescence staining. In wells where BBG3 was included during docetaxel treatment, 3G4 (10 μg/mL) was added and incubated with the cells for 1 hour before staining. Live cell staining was done by growing cells on chamber slides, washing them gently with PBS, and fixing them with 4% paraformaldehyde in PBS at room temperature for 15 minutes. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and incubated with Texas red–labeled phalloidin and FITC-labeled goat anti-mouse antibody for 1 hour at room temperature. To examine DNA fragmentation, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a Coolsnap digital camera mounted on a Nikon microscope and processed with MetaVue software (Universal Imaging Corporation, Downingtown, PA).

Immunofluorescence double staining and quantification of phosphatidylserine exposure *in vivo*. Direct staining of tissue sections by 3G4 does not distinguish between phosphatidylserine that is on the internal versus external surface of the plasma membrane. Therefore, an *in vivo* technique is used where 3G4 is injected into tumor-bearing mice and the 3G4 that binds to externalized phosphatidylserine in the form of phosphatidylserine-protein complexes is identified by indirect immunohistochemistry. Severe combined immunodeficient (SCID) mice bearing orthotopic MDA-MB-435 tumors (~350 mm^3) were treated i.p. with a single dose of docetaxel at 5 or 10 mg/kg or vehicle alone and externalized phosphatidylserine was detected 48 hours after therapy by 3G4 as described (6). Briefly, mice were injected i.v. with 100 μg 3G4. One hour later, mice were anesthetized, exsanguinated, and perfused with heparinized saline. Major organs and tumors were removed and snap frozen. Frozen sections were fixed with 4% paraformaldehyde and blocked with PBS containing 10% bovine serum. 3G4-positive vessels were stained using biotinylated goat anti-mouse IgG followed by FITC-streptavidin. Vascular endothelium was stained using rat anti-mouse CD31 antibody followed by Texas red–labeled goat anti-rat IgG. Single images, taken with appropriate filters for FITC (green) and Texas red (red) fluorescence, respectively, were captured and analyzed. Vessels with exposed anionic phospholipids were identified by yellow fluorescence on merged images. Ten random 0.079 mm^2 fields were evaluated from each section with five tumors in each group. Expression of anionic phospholipids was expressed as the average percentage of 3G4-positive vessels of all CD31-positive vessels identified.

Orthotopic implantation of MDA-MB-435 breast tumor cells. MDA-MB-435 tumor cells were detached from subconfluent cultures by briefly exposing them to 0.25% (w/v) trypsin and 0.02% EDTA. The harvested cells were then washed once in serum-free medium and resuspended in Hanks' balanced buffer. Cell viability was >95% as determined by trypan blue dye exclusion. SCID mice were anesthetized with methoxyflourane inhalation and a small incision was made in the skin over the lateral thorax ~1 cm below the right forelimb. The mammary fat pad was exposed and 2×10^6 MDA-MB-435 cells in 0.1 mL buffer were injected. The incision was closed with surgical clips and mice were allowed to recover. Procedures were conducted in accordance with institutional guidelines.

Treatment of mice bearing orthotopic MDA-MB-435 breast tumor xenografts. Six days after tumor cell injection, when palpable tumors were present in all animals, mice were randomly divided into six groups. The mice were treated i.p. twice a week as follows: group 1, 3G4 (100 μg); group 2, docetaxel (10 mg/kg); group 3, 3G4 (100 μg) plus docetaxel (10 mg/kg); group 4, docetaxel (5 mg/kg); group 5, 3G4 (100 μg) plus docetaxel (5 mg/kg); group 6, control antibody BBG3 (100 μg). Body weight and tumor sizes were assessed twice a week. The general clinical status of the animals was assessed every day. Tumor volume was calculated according to the formula $\pi / 6 \times D \times d^2$, where D is the larger tumor diameter and d is the smaller tumor diameter measured in two perpendicular directions.

Experimental metastatic model of MDA-MB-435 tumor. Female *nu/nu* mice, ages 6 to 8 weeks, were injected with 10^6 MDA-MB-435 cells in 0.1 mL DMEM into a tail vein. Five days after tumor cell injection, mice were randomly separated into four groups of 10 mice. Mice were treated twice a week as follows: group 1, 3G4 (100 μg); group 2, docetaxel (5 mg/kg); group 3, 3G4 (100 μg) plus docetaxel (5 mg/kg); group 4, control antibody BBG3 (100 μg). Treatments were continued for 6 weeks. Eight weeks after injection of tumor cells, the animals were sacrificed and their lungs were removed and fixed in Bouin's fixative. After fixation, the tumor colonies on the lung surface appear white, whereas the normal lung tissue appears brown. The number of tumor colonies on the surface of each lung was counted under a dissecting microscope.

Relative tumor vascularity assessed by an FITC-dextran perfusion assay. Mice bearing orthotopic MDA-MB-435 human breast tumors of volume ~200 mm^3 were treated i.p. twice a week as follows: group 1, 3G4 (100 μg); group 2, docetaxel (10 mg/kg); group 3, 3G4 (100 μg) plus docetaxel (10 mg/kg); group 4, control antibody BBG3 (100 μg). There were five mice in each group. Two weeks after treatment, when divergence in tumor growth between the treatment groups was clearly evident, 0.2 mL of 25 mg/mL FITC-dextran (molecular weight, M_r 2,000,000; Sigma Chemical, Co.,

St. Louis, MO) was injected systemically into the lateral tail vein of each mouse and allowed to circulate for 20 minutes. Mice were then sacrificed and blood samples were collected. Tumors were resected and weighed. To normalize for dilution caused by the difference in tumor sizes, 1 mL of 1:10 dispase was added per 0.5 g tissue. Tumors were incubated overnight in the dark at 37°C. The tissue was homogenized, centrifuged at 3,000 × *g* for 10 minutes, and supernatant was collected and stored in the dark. The fluorescence of the supernatant was measured in a fluorometer (Turner model 450; excitation at 492 nm, measurement at 515 nm). The ratio of tumor fluorescence/plasma fluorescence reflects the extent of tumor perfusion.

Immunofluorescence double staining and quantification of apoptosis of tumor and endothelial cells. Frozen sections of tumors from mice treated as above were fixed and incubated for 18 hours with rat monoclonal anti-CD31 antibody (1:100) at 4°C, rinsed with PBS, and incubated with goat anti-rat IgG conjugated to Texas red for 60 minutes at ambient temperature in the dark. Sections were then washed with PBS and fixed with 4% (w/v) paraformaldehyde for 10 minutes. Terminal dUTP-mediated nick end labeling (TUNEL) staining was done to detect fragmented DNA using a commercial kit (Promega Corp., Madison, WI) according to the manufacturer's instructions with the following modifications. Sections were permeabilized by incubating with 0.2% Triton X-100 in PBS for 15 minutes. The sections were incubated with equilibration buffer, drained, and a reaction medium containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase was added. The sections were incubated in a humidified chamber for 1 hour at 37°C in the dark. The reaction was terminated by immersing the sections in 2× SSC [30 mmol/L NaCl, 3 mmol/L sodium citrate (pH 7.2)] for 15 minutes, followed by three washes to remove unincorporated fluorescein-dUTP. To quantify apoptotic endothelial cells, nuclei were stained with DAPI. Endothelial cells were identified by red fluorescence and DNA fragmentation was detected by localized green fluorescence. Yellow fluorescence within the nucleus (visualized by DAPI stain) indicated apoptotic endothelial cells. Images were captured and processed as described above. Apoptotic endothelial cells in 10 random 0.079 mm^2 fields were counted at ×200 magnification. The average percentage of TUNEL-positive endothelial cells of all CD31-positive endothelial cells was calculated for five different tumors. To quantify tumor cell apoptosis, TUNEL-positive tumor cells in 10 random 0.079 mm^2 fields were counted. The average number of TUNEL-positive tumor cells per square millimeter for five different tumors was calculated.

Quantification of microvascular density. To quantify tumor vessel counts, frozen sections of tumors from treated mice section were stained with anti-CD31 antibody as previously described (28). Ten random 0.079 mm^2 fields at ×200 magnification for each tumor were captured using a Coolsnap digital camera mounted on a Nikon microscope and Metavue software. The images were used to count microvessel-like structures consisting of endothelial cells that were stained with the anti-CD31 antibody as previously described (29). Five tumors from each group were analyzed. The microvascular density (MVD) was expressed as the number of vessels per square millimeter.

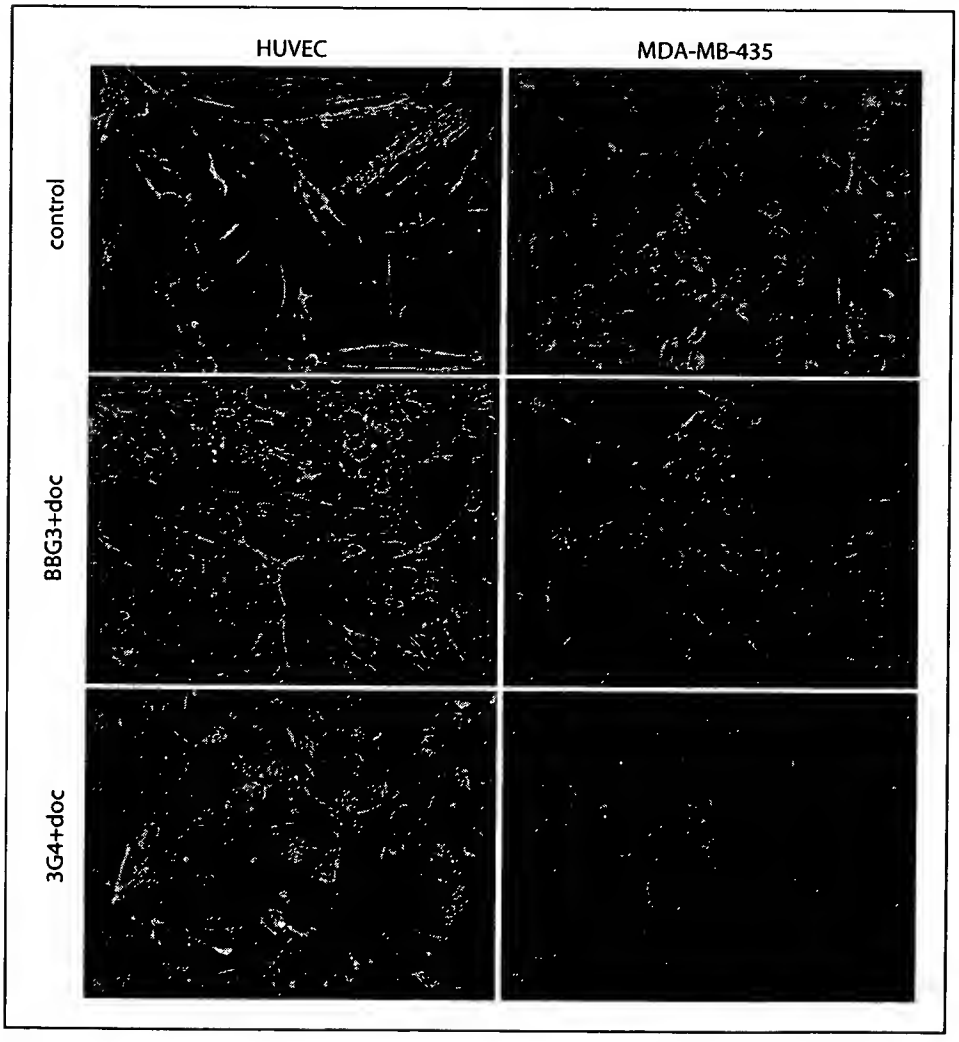

Figure 1. Exposure of anionic phospholipids induced by subtoxic concentrations of docetaxel on HUVEC cells but not on MDA-MB-435 tumor cells *in vitro*. HUVEC or MDA-MB-435 cells were grown on chamber slides and were treated with docetaxel (20 pmol/L) in the presence of 3G4 or control antibody BBG3 (10 μg/mL) for 24 hours. Exposure of anionic phospholipids was then examined by live cell immunofluorescence staining as described in Materials and Methods. Externalized anionic phospholipids was stained with 3G4 in green (FITC), cytoskeleton in red (Texas red–labeled phalloidin), and nuclei in blue (DAPI). No exposure of anionic phospholipids was seen on cells not treated with docetaxel or on cells treated with 3G4 alone (not shown). Strong exposure of anionic phospholipids was seen on nearly 100% of HUVECs treated with docetaxel at 20 pmol/L (*middle left*). More than 90% of the cells were nonapoptotic as judged by the absence of morphologic changes in the nuclei and were as capable of proliferation as untreated cells after removal of the drug. Capping of anionic phospholipids was observed on cells treated with docetaxel plus 3G4 (*bottom left*), whereas a punctate pattern exposure was observed on cells treated with docetaxel plus control antibody BBG3 (*middle left*) or with docetaxel alone (not shown). In contrast, no exposure of anionic phospholipids was observed on MDA-MB-435 tumor cells treated with docetaxel at 20 pmol/L in the presence or absence of 3G4 (*bottom right*) or BBG3 (*middle right*).

Statistical analyses. The tumor volume, number of metastatic tumor colonies, tumor MVD, and apoptotic indices of tumor and endothelial cells were compared by Student's paired *t* test. $P < 0.05$ was considered significant.

Results

Exposure of anionic phospholipids is induced by subtoxic concentrations of docetaxel on human umbilical vascular endothelial cells but not on MDA-MB-435 tumor cells *in vitro*. We first determined whether subtoxic concentrations of docetaxel can induce externalization of anionic phospholipids on human umbilical vascular endothelial cells (HUVEC) and MDA-MB-435 cells *in vitro*. Conditions were selected (20 pmol/L docetaxel, 24 hours, 37°C) that caused a <10% inhibition of cell proliferation when the drug was removed and the cells cultured for a further 72 hours and assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Neither cell type was apoptotic: Morphologic changes in the nuclei were not observed (Fig. 1) and <10% of the cells displayed apoptosis markers, active caspase 3, or fragmented DNA (TUNEL). Under these nontoxic conditions, docetaxel induced exposure of anionic phospholipids on nearly 100% of HUVECs. Small membrane blebs that were intensely stained by 3G4 were visible and distributed in punctate pattern over the surface of the HUVEC (Fig. 1, *middle left*). Identical staining patterns were observed with 3SB, a phosphatidylserine-specific antibody (30), and with annexin V (not shown). When 3G4 was included in the culture medium together with docetaxel, a different staining pattern was observed. The membrane blebs congregated into "caps" (Fig. 1, *bottom left*). This redistribution was not observed with cells coincubated with docetaxel and the isotype-matched control antibody, BBG3 (Fig. 1, *middle left*). Incubation of cells with 3G4 in the absence of docetaxel caused no detectable externalization of anionic phospholipids (data not shown).

In contrast to HUVECs, no exposure of anionic phospholipids was detected on MDA-MB-435 cells cultured with 20 pmol/L docetaxel in the presence or absence of 3G4 (Fig. 1, *right*). Even when the concentration of docetaxel was increased to 200 pmol/L, no externalization of phosphatidylserine was visible (not shown). Thus, MDA-MB-435 cells do not seem to externalize anionic phospholipids in response to low concentrations of docetaxel.

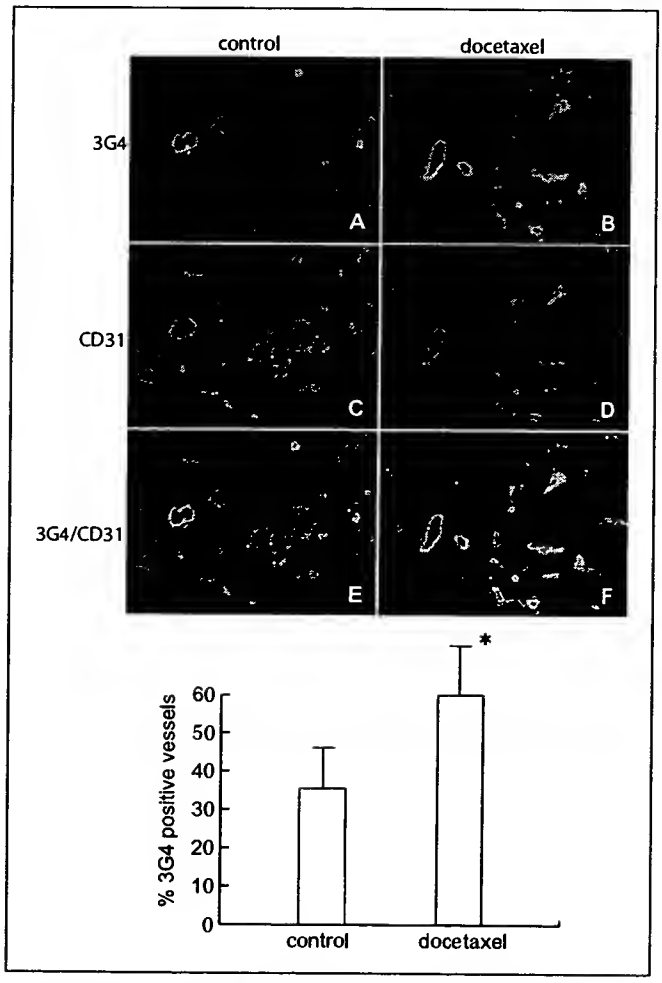

Figure 2. Exposure of anionic phospholipids is enhanced by docetaxel on tumor vascular endothelial cells *in vivo*. SCID mice bearing orthotopic MDA-MB-435 tumors (~350 mm^3) were injected i.p. with docetaxel (10 mg/kg) or vehicle alone. Forty-eight hours later, exposure of anionic phospholipids on tumor vascular endothelium was determined by injecting 3G4 i.v. and perfusing the mice 1 hour later. Tumors were harvested and frozen sections cut and analyzed. Localized 3G4 was stained green with FITC-labeled anti-mouse immunoglobulin (*A*, *B*). Vessels were stained in red with rat anti-CD31 antibody followed by Texas red–labeled anti-rat immunoglobulin (*C*, *D*). The yellow color in merged images identifies 3G4-positive tumor vessels (*E*, *F*). Representative images are shown. *A*, *C*, *E*, control vehicle–treated group; *B*, *D*, *F*, docetaxel-treated group. The percentage of 3G4-positive vessels was calculated from the number of 3G4-stained vessels relative to the number of CD31-positive vessels in 10 random 0.079 mm^2 fields at ×200 magnification. *Columns*, average percentages; *bars*, SE ($n = 5$). The percentage of 3G4-positive tumor vessels increased from 35% to 60% after a single injection of docetaxel (*$P < 0.01$).

Enhancement of exposure of anionic phospholipids on tumor blood vessels by docetaxel. We next determined whether docetaxel can enhance externalization of anionic phospholipids on tumor vascular endothelial cells *in vivo*. Mice bearing orthotopic MDA-MB-435 breast tumors were given a single i.p. injection of docetaxel (10 mg/kg). Exposure of anionic phospholipids on tumor vessels was determined 24 to 72 hours later by injecting 3G4 and using an immunofluorescence double-staining technique to detect 3G4 that had specifically localized to the vascular endothelium of tumor and normal tissues (Fig. 2).

A marked increase in the exposure of anionic phospholipids on tumor vessels was observed 24 to 72 hours after docetaxel treatment, with peak levels being obtained at 48 hours posttreatment. The average percentage of 3G4-positive tumor vessels in mice treated with 10 mg/kg docetaxel was 60 ± 12%, whereas with 5 mg/kg docetaxel it was 49 ± 6%. In vehicle-treated tumors, the average percentage of 3G4-positive vessels was 35 ± 7% ($P < 0.01$). The majority of 3G4-positive vascular endothelial cells were not apoptotic. Only 4.0% of endothelial cells in tumors from mice treated with docetaxel (10 mg/kg) were apoptotic as judged by TUNEL staining (see below). This result is consistent with the finding above that docetaxel treatment of HUVEC cells causes exposure of anionic phospholipids without causing apoptosis. In docetaxel-treated tumors, occasional regions of nonvascular 3G4 staining were observed (Fig. 2*F*, *green*). These cells are probably apoptotic tumor cells.

No exposure of anionic phospholipids was detected on blood vessels in any of the normal organs from docetaxel-treated mice. The normal organs examined were heart, lung, brain, kidney, liver, spleen, testis, and pancreas (results not shown). These results suggest that tumor vascular endothelial cells are more sensitive than normal endothelial cells to docetaxel treatment.

Inhibition of growth of orthotopic breast tumors in mice. The effect of 3G4 in combination with docetaxel on tumor growth was examined in the orthotopic MDA-MB-435 human breast cancer model. Six days after tumor cell inoculation, mice were treated i.p. with 3G4 (100 μg), BBG3 (100 μg), docetaxel (10 mg/kg), or the combination of 3G4 (100 μg) and docetaxel (10 mg/kg) twice a week for 3 weeks (Fig. 3*A*). Tumor growth in BBG3 recipients was indistinguishable from that in untreated mice (result not shown). Tumor growth was almost completely suppressed by the combination therapy. By day 31, tumor growth was suppressed by 93% with the combination therapy compared with 50% for 3G4 ($P < 0.005$) and 70% for docetaxel ($P < 0.01$). We also tested the effects of the combination of 3G4 with docetaxel at a lower dose (5 mg/kg, biweekly), which caused no loss in body weight. The combination therapy inhibited tumor growth by 73% compared with 50% and 43% for 3G4 alone ($P < 0.01$) and docetaxel alone ($P < 0.01$), respectively (Fig. 3*A*). These results show that 3G4 significantly enhances the therapeutic efficacy of docetaxel for the treatment of orthotopic MDA-MB-435 human breast tumors in mice.

Lack of enhancement of docetaxel toxicity in mice. Body weight was used as a surrogate indicator of the health status of the mice (Fig. 3*B*). We found that 3G4-treated mice gained weight at the same rate as did BBG3 control mice as in prior studies (10). Docetaxel (10 mg/kg, biweekly) caused a marked loss of body weight (~20%), indicating that this dose was close to the maximum tolerated dose in our experimental model. Animals regained body weight when the docetaxel treatment was stopped. Importantly, no enhancement in toxicity was seen when 3G4 was combined with docetaxel. Mice treated with the combination lost and regained body weight in an identical manner to mice treated with docetaxel alone (Fig. 3*B*). Histologic examination of the lung, liver, heart, brain, intestine, stomach, and kidney from mice treated with the combination revealed no differences from mice treated with docetaxel alone.

Reduction in tumor colonies in the lungs of mice. The effects of treatment with 3G4 alone or in combination with docetaxel were examined in an experimental model of metastasis in which MDA-MB-435 human breast cancer cells are injected i.v. into a tail vein of *nu/nu* mice. The breast cancer cells form tumor colonies in the lungs of the mice. Treatment was begun 5 days after tumor cell injection. The experiment was terminated on day 56 when the BBG3 control group developed symptoms of respiratory disease. An average of 31 ± 10 tumor colonies was observed on the surface of the lungs in BBG3 control mice. Treatment with 3G4, docetaxel, or the combination therapy reduced the average numbers of tumor colonies on the surface of the lungs to 5.5 ± 2.7, 7.0 ± 4.9 and 2.3 ± 2.0, representing a decrease of 82%, 78%, and 93%, respectively (Fig. 4). The combination therapy was significantly more effective than either agent alone ($P < 0.05$). All 10 mice in the BBG3 control group had tumor foci on the surface of their lungs, compared with only 5 of 10 in the combination treated group, 8 of 10 in the 3G4 treated group, and 9 of 10 in docetaxel-treated group. Histologic examination of paraffin sections of the lungs of mice treated with the combination revealed that 5 of 10 mice having no surface colonies had no microscopic evidence of tumor in their lungs. In the remaining five mice treated with the combination, occasional small nests of tumor cells were seen in the lungs. All mice treated with control BBG3 antibody or either agent alone had tumor colonies in their lungs. The sizes of the colonies were also markedly smaller in the combination-treated group. Because the treatment was started 5 days after tumor cell injection, tumor colonies had most likely formed in the lungs before treatment began. It is, therefore, likely that the antitumor effect is due to inhibition of the growth of lung colonies rather than to inhibition of colonization itself.

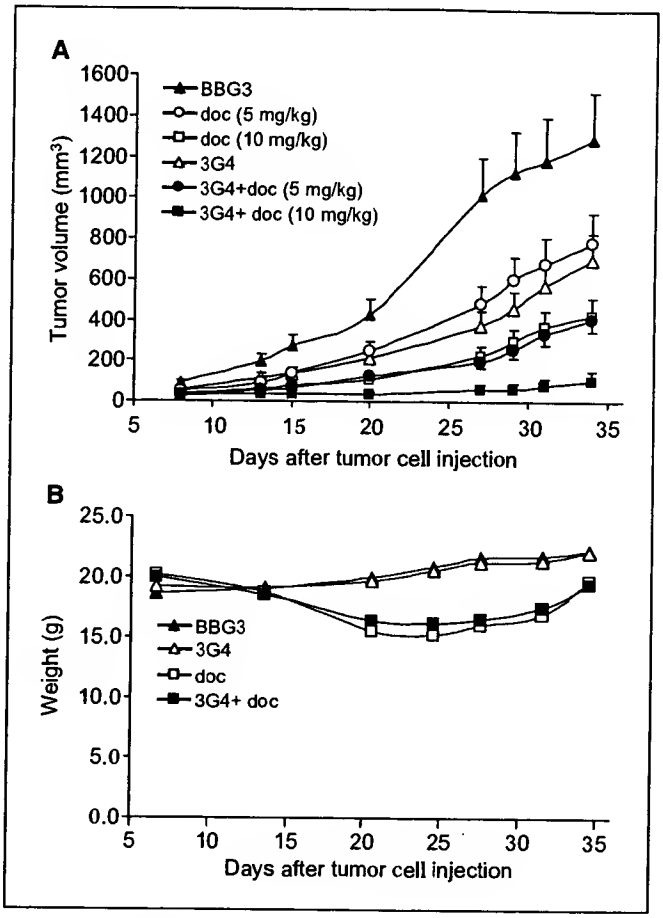

Figure 3. Enhanced inhibition of orthotopic human breast tumors by 3G4 plus docetaxel. Four groups of 10 mice bearing orthotopic MDA-MB-435 human breast tumors were treated 6 days after tumor cell injection with biweekly i.p. injections of 3G4 (100 μg), docetaxel (5 or 10 mg/kg), and the combination of 3G4 (100 μg) plus docetaxel (5 or 10 mg/kg). Another group of mice received the control antibody, BBG3 (100 μg). Treatments were continued for 3 weeks. Tumor volumes were measured twice weekly and the body weights of the mice were recorded regularly. *A*, effects on tumor growth. The combination therapy was significantly more effective than either agent alone. Tumor growth in BBG3 recipients was indistinguishable from that in untreated mice in multiple experiments (result not shown). *Points*, mean tumor volumes in groups of mice; *bars*, SE. *B*, lack of effect of 3G4 on toxicity-dependent weight loss caused by docetaxel. The 3G4-treated group gained weight at the same rate as the control BBG3-treated group. Animals treated with docetaxel (10 mg/kg, biweekly) experienced a marked (~20%) body weight loss but regained weight after treatment was stopped on day 28. There is no significant difference in weight changes between the groups of mice treated with the combination versus docetaxel alone. *Points*, average body weights (g).

Enhanced antivascular effects of 3G4 plus docetaxel on human breast tumors in mice. The effect of the combination therapy on the functionality of tumor vasculature was examined by using a FITC-dextran perfusion assay. Mice bearing established orthotopic MDA-MB-435 human breast tumors (200 mm^3) were treated i.p. with 3G4, docetaxel, or combination therapy for 2 weeks. Two days after the last treatment, FITC-dextran was injected into the lateral tail vein and allowed to equilibrate throughout the vascular compartment. The fluorescence in the tumors is determined by tumor blood volume and perfusion. The majority of the dextran remains intravascular because of its large size (2×10^6 Da;

ref. 31). Treatment with 3G4 or docetaxel, alone or in combination, significantly reduced tumor perfusion compared with the BBG3 control treatment (Fig. 5*A*). The combination of 3G4 plus docetaxel was the most effective. It reduced tumor perfusion by 75% compared with 56% and 59% for 3G4 and docetaxel alone, respectively ($P < 0.01$ for combination versus each agent alone).

The MVD was significantly reduced in tumors of mice from all treated groups compared with the BBG3 control group ($P < 0.01$). The combination of 3G4 plus docetaxel was the most effective. It reduced tumor MVD by 71% compared with 62% for 3G4 and 60% for docetaxel alone ($P < 0.05$ for combination versus each agent alone; Fig. 5*B*). These results indicate that the combination of 3G4 plus docetaxel has strong antivascular activity.

Enhanced apoptosis of tumor vascular endothelial cells and tumor cells in mice treated with 3G4 plus docetaxel. We determined whether vascular endothelial cells and tumor cells in mice treated with 3G4, docetaxel, or the combination become apoptotic as a result of the treatment. Mice bearing orthotopic MDA-MB-435 tumors were treated with 3G4, docetaxel, or the combination twice a week for 2 weeks. Forty-eight hours after the last treatment, tumors were removed and endothelial cells were examined using immunofluorescence double staining with anti-CD31 and TUNEL. The combination of 3G4 and docetaxel caused a significantly greater percentage (8.9%) of tumor endothelial cells to become apoptotic than in mice treated with 3G4 (5.3%) or docetaxel (4.5%) or BBG3 (0.8%) alone ($P < 0.01$). These results suggest that the combination therapy significantly enhances apoptosis of tumor endothelial cells, which might be responsible for the reduction in tumor MVD and blood perfusion (Fig. 6*A*).

The percentage of apoptotic tumor cells was significantly increased from 5% in BBG3 controls to 10%, 12%, and 20% posttherapy with 3G4, docetaxel, or the combination of 3G4 and docetaxel, respectively (Fig. 6*B*). The combination of 3G4 and docetaxel produced significantly more tumor cell apoptosis than did each agent alone ($P < 0.05$). Clusters of apoptotic tumor cells were seen surrounding apoptotic endothelial cells in 3G4 and the combination-treated tumors, suggesting that the markedly increased apoptosis of tumor cells is secondary to vascular damage caused by the therapy.

Discussion

The major findings of the present study are that the vascular targeting antibody, 3G4, significantly enhances the therapeutic efficacy of docetaxel against the growth and dissemination to the lungs of MDA-MB-435 human breast tumors in mice without concomitant increase in host toxicity.

Docetaxel is the most important cytotoxic drug currently available for treating breast cancer; approximately half of all patients with the disease receive docetaxel therapy (32). It is also becoming

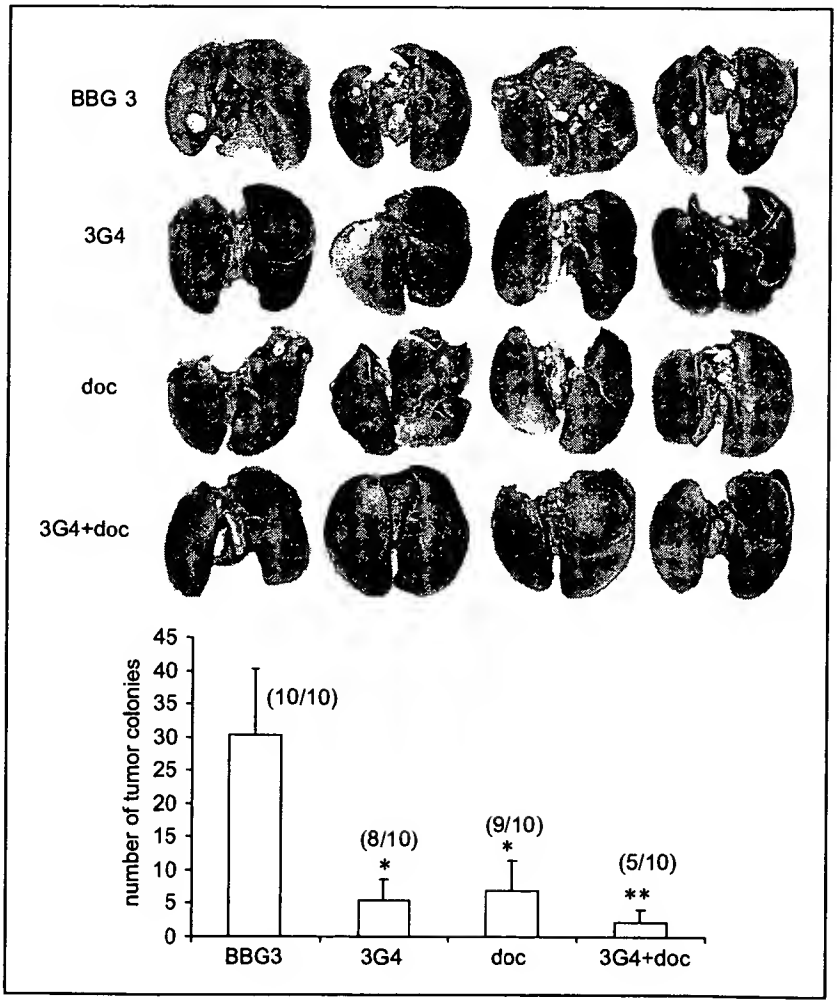

Figure 4. Enhanced inhibition of the establishment and growth of human breast tumors in the lungs of mice treated with 3G4 plus docetaxel. *Nu/nu* mice were injected i.v. with 1×10^6 MDA-MB-435 human breast tumor cells into a tail vein. Treatment with 3G4 and/or docetaxel began 5 days after tumor cell injection. The number of tumor colonies in the lungs was determined on day 56. The number of surface tumor colonies was counted for 10 mice from each group and the average was calculated. *Columns*, mean; *bars*, SE. The numbers in parentheses are the number of mice in each group that developed tumor colonies on the surface of their lungs. Representative pictures of the lungs are shown. In mice treated with control antibody BBG 3, many large tumor colonies were seen on the surface of the lungs. In the 3G4 and docetaxel groups, relatively few, small colonies were observed. Yet, fewer and smaller colonies or no colonies at all were seen in the combination group. Individually, 3G4 and docetaxel significantly inhibited the formation and growth of pulmonary tumor colonies (*$P < 0.05$, compared with control). The combination therapy was significantly more effective than either single agent alone (**$P < 0.05$, compared with single agent).

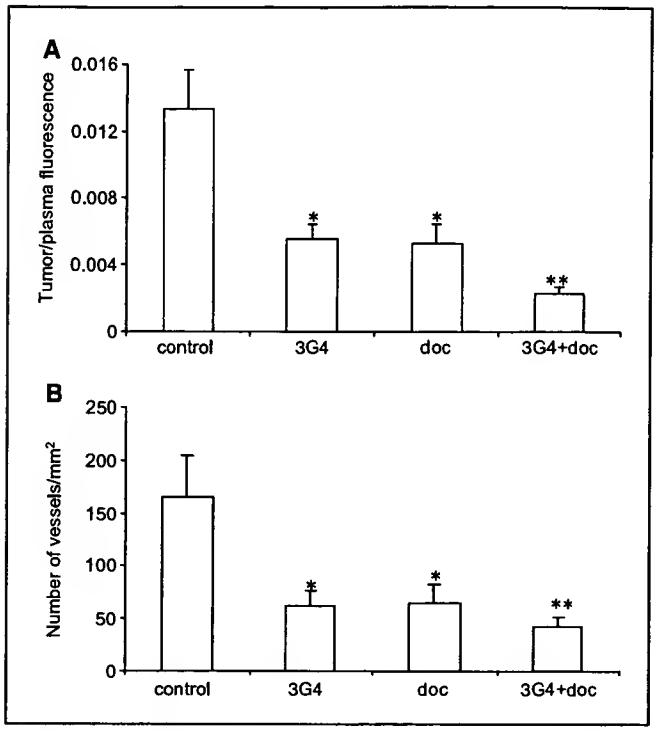

Figure 5. Enhanced antivascular effects of 3G4 plus docetaxel on human breast tumors in mice. Mice bearing orthotopic MDA-MB-435 tumors (~200 mm^3) were treated i.p with 3G4 (100 μg) or docetaxel (10 mg/kg) alone or in combination twice a week for 2 weeks. Other mice received BBG3 control antibody (100 μg). The antivascular effects of the combination therapy were evaluated by measuring functional blood volume and MVD in the tumors. *A*, FITC-dextran perfusion assay was done as described in Materials and Methods. The ratio of tumor/plasma fluorescence is a measure of tumor blood volume and perfusion. *Columns*, mean; *bars*, SE (n = 5). *B*, tumor MVD. Tumor sections were stained with anti-CD31 antibody. *Columns*, MVD expressed as the mean number of vessels per square millimeter; *bars*, SE. Data were obtained by analyzing 10 random 0.079 mm^2 fields on each section at ×200 magnification with five tumors in each group. In both (*A*) and (*B*), the combination treatment was significantly more effective than each agent alone (**$P < 0.01$). The individual treatments significantly reduced tumor/plasma fluorescence and MVD relative to the BBG3 control group (*$P < 0.01$).

increasingly important for treating ovarian and prostate cancer (33). However, the dose of docetaxel that can be given to patients is limited by the toxicity of the drug, principally neurotoxicity, fluid retention, and neutropenia (34, 35). There is a pressing need for combination therapies to use with docetaxel that improve its efficacy without exacerbating its toxicity. Combining docetaxel with agents that attack or destroy tumor vessels has yielded promising results (36–39). Numerous studies have shown that the therapeutic efficacy of docetaxel can be significantly enhanced when combined with inhibitors of angiogenesis (40–42) and VTAs (43).

Here, we explored the possibility that 3G4 might cooperate with docetaxel to give improved antitumor effects. We found that the 3G4/docetaxel combination was significantly superior to the individual drugs at treating both established human breast tumors growing in the mammary fat pads of mice and against tumors that had disseminated to the lungs. These results are not explicable by direct exposure of anionic phospholipids on tumor cells because the MDA-MB-435 tumor cells used in our studies did not expose anionic phospholipids in response to subtoxic concentrations of docetaxel.

The first possible mechanism of the improved antitumor effects of the combination is that docetaxel induces exposure of anionic phospholipids on tumor vasculature, as shown herein, leading to increased binding of 3G4 and destruction of tumor vessels, probably through Fc-dependent mechanisms (antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, or antibody-mediated macrophage phagocytosis as observed previously; refs. 44, 45). In support of this mechanism, we found that the percentage of 3G4-positive vessels in orthotopic MDA-MB-435 tumors was increased by ~70% (from 35% to 60%) after a single injection of docetaxel (10 mg/kg); this was accompanied by increased endothelial cell apoptosis in tumors from mice treated with the combination and a significantly greater impairment in tumor vascular function as judged by reduction in tumor perfusion and in MVD.

In vitro studies showed that HUVEC cells that had been treated with a subtoxic concentration of docetaxel consistently showed exposure of anionic phospholipids without undergoing apoptosis. The exposed anionic phospholipids also appeared to be gathered into clusters or caps in the presence of 3G4 as opposed to the punctate membrane distribution seen in the absence of 3G4. Possibly, docetaxel causes anionic phospholipids to shuttle between the inner and outer leaflets of the plasma membrane and becomes trapped and cross-linked on the cell surface by the 3G4. Exposure of anionic phospholipids on nonendothelial cells has been reported to be caused by docetaxel (46). The mechanism by which docetaxel induces exposure of anionic phospholipids on viable endothelial cells may relate to its ability to generate reactive oxygen species (47–49). Reactive oxygen species are strong inducers of externalization of anionic phospholipids. They are thought to act either by oxidizing lipids leading to local disturbances in lipid packing or by oxidizing lipid or ion transport molecules (50). These perturbations may induce Ca^{2+} influxes that inhibit aminophospholipid translocase or activate phosphatidylserine exporters, resulting in the bulk movement of phosphatidylserine to the external cell surface. The lack of docetaxel-induced exposure of phosphatidylserine on MDA-MB-435 tumor cells contrasts with the exposure on HUVECs (this study). Also, in contrast with many types of tumor cells (51), MDA-MB-435 cells lack constitutive exposure of phosphatidylserine, suggesting that they may have different phosphatidylserine regulation as has been reported for other tumor cell types (52).

The second possible mechanism that could account for the increased antitumor activity of the combination therapy is that 3G4 and docetaxel have complementary antitumor effects. 3G4, like other VTAs, would be expected to be more active in the poorly vascularized and poorly perfused tumor regions. Also, phosphatidylserine, the major target for 3G4, is induced by such stress conditions in tumor microenvironment as hypoxia and acidosis, which are associated with poor vascularization and/or poor perfusion in tumors. Evidence to support this mechanism comes from histologic examination of 3G4-treated tumors, which shows extensive hemorrhagic necrosis through the central region of the tumor, surrounded by a rim of viable tissue. In contrast, docetaxel, as a cycle-selective cytotoxic drug, would be expected to be more effective in the well-vascularized regions where tumor cells divide most rapidly and have limited activity against poorly perfused regions where tumor cells cycle slowly. It is well accepted that complementary killing of different regions of tumors is a general mechanism explaining the enhanced activity of antivascular drugs and conventional cytotoxins.

In contrast to vessels in tumors, no externalization of anionic phospholipids was induced by a single dose of docetaxel (10 mg/kg)

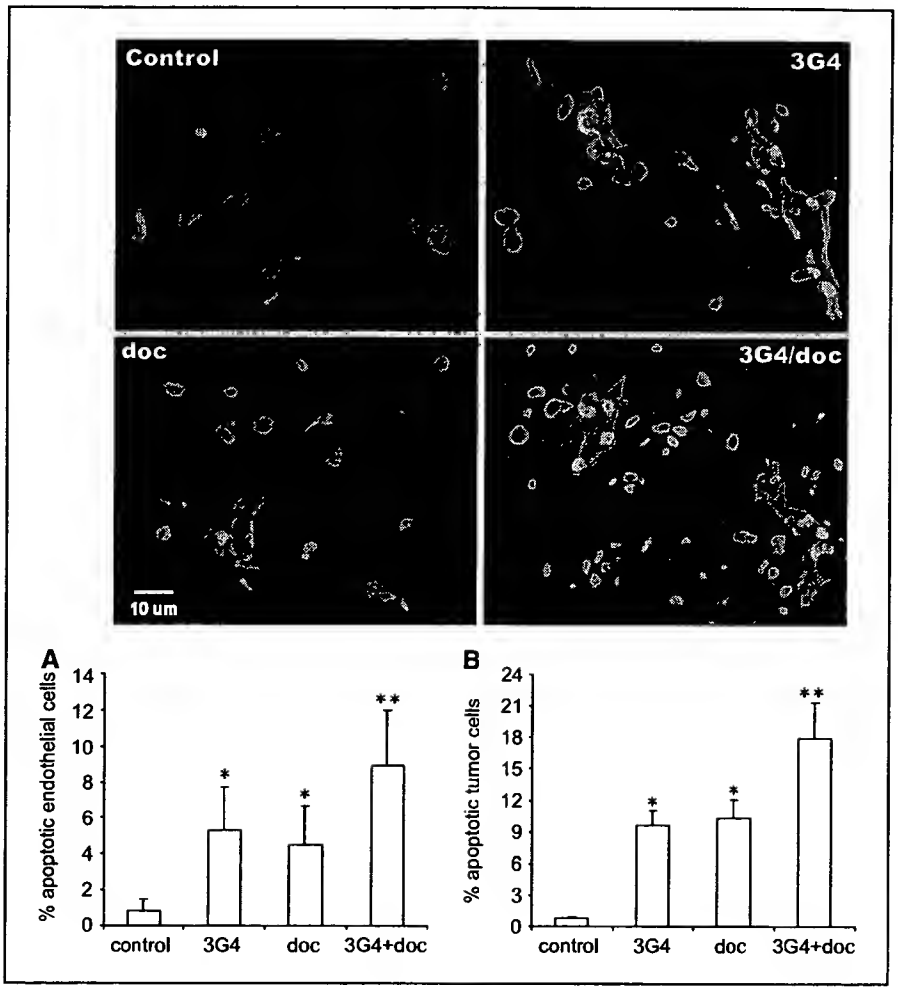

Figure 6. Apoptosis of tumor vascular endothelial cells and tumor cells induced by docetaxel plus 3G4 treatment in mice. Mice were treated with 3G4 (100 μg) or docetaxel (10 mg/kg) alone or in combination biweekly i.p. for 2 weeks. Other mice received BBG3 control antibody (100 μg). Tumors were harvested and processed for immunofluorescence double staining of CD31 (*red*) and TUNEL (*green*). Double labeling of endothelial cells undergoing apoptosis results in localized yellow fluorescence in overlapped images. Endothelial cell (*A*) and tumor cell (*B*) apoptosis is significantly increased in the tumors of mice treated with 3G4 or docetaxel alone compared with BBG3 control–treated mice (*$P < 0.01$). The combination treatment causes significantly more apoptosis of endothelial cells and tumor cells than the individual treatments (**$P < 0.01$). Clusters of apoptotic tumor cells surrounding the apoptotic vessels are evident in the combination-treated tumors. Apoptotic cells in 10 random 0.079 mm^2 fields were counted for each section at ×200 magnification. Five sections from each of five tumors per group were analyzed (see Materials and Methods). *Columns*, mean; *bars*, SE.

on the vascular endothelium of any of the normal organs that were examined, including the brain, heart, kidney, liver, lung, pancreas, and spleen. These results suggest that the conditions in the tumor microenvironment might play an important role in docetaxel-induced exposure of anionic phospholipids on tumor vascular endothelium. There are several possible reasons why tumor vessels might be more sensitive to docetaxel than normal vessels. First, tumor vessels are structurally deficient, having tortuous, leaky, thin walls, and insufficient supportive pericytes (53). Tumor vessels are inherently more sensitive than normal vessels to chemotherapeutic drugs, radiotherapy, and VTAs (54). Second, in contrast to tumor vascular endothelial cells, which are generally active and proliferating, endothelial cells in normal tissues are quiescent and nondividing, and therefore not sensitive to antimitotic drugs such as docetaxel. The fact that exposure of anionic phospholipids was induced on subconfluent dividing HUVEC *in vitro* supports this view. Our finding that 3G4 plus docetaxel was no more toxic than was docetaxel alone agrees with the lack of exposure of anionic phospholipids on the vascular endothelium in normal tissues. However, it is important to note that the tumor-bearing mice in our study were otherwise normal, young mice. Further, studies are needed to determine whether 3G4 might negatively impact vessels in inflamed tissues or healing wounds where docetaxel might possibly induce phosphatidylserine exposure on the activated/regenerating endothelium. If so, patients who have undergone recent surgery or who have inflammatory disease might not be candidates for 3G4 plus docetaxel therapy. As observed previously (10), 3G4 was itself not toxic. We did not observe signs of antiphospholipid syndrome similar to those reported for certain anticardiolipin antibodies with reactivity against β2-glycoprotein I (55).

A chimeric version of 3G4 (Tarvacin) has been developed and is scheduled to enter clinical trials in cancer patients. The present study suggests that clinical evaluation of Tarvacin with docetaxel is warranted. Because the antibody is long-lived, it could be administered at the same time as docetaxel using normal scheduling. We are currently investigating whether the enhanced therapeutic efficacy with 3G4 and docetaxel observed with the MDA-MB-435 human breast tumor extends to other tumor models and to other conventional therapies.

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